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Evidence for a Role of the Gill in Osmotic Regulation in the Horseshoe Crab, Limulus polyphemus Linnaeus

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EVIDENCE FOR A ROLE OF THE GILL IN OSMOTIC REGULATION IN THE HORSESHOE CRAB, *LIMULUS POLYPHEMUS* LINNAEUS

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This thesis is submitted in partial fulfillment of
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ABSTRACT

The gill of the horseshoe crab, Limulus polyphemus consists of five paired stacks of thin chitinous lamellae, called book gills, each book containing about 150 lamellae. An oval-shaped region in the center of each lamella is visibly distinct from the outer, or peripheral region and covers about one-third of a lamella. The ultrastructure of these two regions was examined and found to contain morphologically distinct areas. Both sides of the blood space in the peripheral region and the dorsal side in the central region are lined with a single layer of thin, undistinguished cells. Cells in the central patch on the ventral, or recovery-stroke side of the blood space, on the other hand, exhibit elaborate infoldings of the basal membrane and interdigitations of lateral membranes with closely-associated and densely-packed mitochondria.

This localized region of the gill lamellae correspondingly contained high levels of $Na^+ + K^+$-dependent ATPase ($Na^+ + K^+$-ATPase) activity. The coxal gland had similarly high levels of enzyme activity whereas the peripheral region and the central dorsal region of the gill lamellae contained very little activity. $Na^+ + K^+$-ATPase content was similar in the five pairs of gill books and no activation occurred in either branchial or coxal gland enzyme with acclimation to reduced environmental salinity.

The presence of ion-transporting cells and relatively high levels of $Na^+ + K^+$-ATPase activity suggests active participation by the gill in ionic and osmotic regulation in the horseshoe crab.

EVIDENCE FOR A ROLE OF THE GILL IN OSMOTIC REGULATION IN THE HORSESHOE CRAB, *LIMULUS POLYPHEMUS* LINNAEUS

INTRODUCTION

The horseshoe crab, Limulus polyphemus Linnaeus, is a chelicerate arthropod which inhabits the western Atlantic from Maine to Mexico (Robertson, 1970; Shuster, 1982). Horseshoe crabs are common sights on sandy beaches along the east coast of the United States and in bays and estuaries where in the spring the crabs migrate to spawn (Teale, 1957; Cavanaugh, 1975; Cohen and Brockman, 1983). Adult Limulus typically inhabit offshore waters but during the spring migration may be found in waters ranging from 50 to 100% seawater and can tolerate salinities as low as 20% SW so long as the change is gradual (McManus 1969). A large and abrupt decrease in salinity can result in ruptured gill lamellae and fatal hemorrhaging due to a rapid intake of water across the highly permeable carapace and gill (Dunson, 1984). The regulatory mechanisms are presumably inadequate to cope with sudden and extreme osmotic stress.

Compared to more fully euryhaline arthropods such as the blue crab, Callinectes sapidus (Engel et al., 1974; Weiland and Mangum, 1975; Mangum and Towle, 1977), Limulus is a weak osmoregulator, maintaining its

blood osmolality and ion levels slightly above those in dilute media (Towle et al., 1982). According to Robertson (1970) and Towle et al. (1982) the blood is isosmotic and slightly hypoionic to the medium at 35 ppt and becomes hyperosmotic below 35 ppt and hyperionic with respect to Na^+ and Cl^- below 32 ppt.

The so-called book gill of the horseshoe crab is attached to modified appendages and consists of five paired stacks of thin chitinous lamellae arranged anterio-posteriorly on the ventral surface of the opisthosoma (Mangum, 1982). Rippling like the pages of a book, hence its name, the gill is ventilated and helps propel the animal forward. Each of the stacks contains approximately 150 lamellae each lamella possessing a central elliptical patch that differs in appearance from the periphery (Mangum, 1982). Similar differentiation occurs in the lamellae of blue crab gills where this patch, larger in posterior than anterior gills, results from a concentration of ion-transporting cells (Copeland, 1964; Neufeld et al., 1980; Cioffi, 1984). Covering roughly half of the ventral surface and containing approximately 1500 lamellae, the horseshoe crab gill provides a large surface area for communication with the external environment and possesses at least one feature, the lamellar patch,

characteristic of transporting branchial epithelia in other taxa.

Transporting epithelial tissue is characterized by cells with extensive basal and lateral membrane infoldings densely packed with mitochondria (Oschman and Berridge, 1971; Cioffi, 1984). Variations on this theme have been found in cells from gills of teleost fish (Philpott and Copeland, 1963; Sargent, 1974; Laurent and Dunel, 1980), and several decapod crustaceans (Copeland, 1964, 1968; Barra et al., 1983; Pequeux et al., 1984), and from excretory organs of crayfish (Peterson and Loizzi, 1974a), fiddler crabs (Schmidt-Nielsen et al., 1968), horseshoe crabs (Towle et al. 1982) and scorpions (Rasmont, et al., 1958).

Localized in these basolateral membranes is the enzyme Na^++K^+-dependent adenosine triphosphatase (Na^++K^+-ATPase) (Ernst, 1972; Peterson & Loizzi, 1974b; Ernst, 1975; Towle et al., 1983), first described by Skou (1957) and now a very useful marker of ion-transporting epithelia (Bonting, 1970; Towle, 1984). Na^++K^+-ATPase is the Na^+ pump that utilizes energy from the hydrolysis of ATP to transport Na^+ against the electrochemical gradient in exchange for a counterion in an approximate ratio of 3 to 2 (Skou, 1965; Glynn and Karlish, 1975 for review). High

specific activities typically exist in specialized ion-transporting tissues such as the gills (Bonting, 1970). The action keeps intracellular Na^+ concentrations low so that Na^+ enters the cell from the external medium down its concentration gradient, possibly coupled to H+ or NH_4^+ efflux (Pequeux & Gilles, 1978; Girard & Payan, 1980; Perry, 1981; Kirschner, 1983; Towle et al., 1984; Towle & Breaux, 1984).

In the gills of euryhaline fishes, Na^++K^+-ATPase activity increases with the onset of either hypo- or hyperosmotic regulation (Epstein et al., 1967; Karnaky, 1980; Borgatti, 1985; Dange, 1985). In osmoregulating invertebrates, Na^++K^+-ATPase activity generally increases in ion-transporting tissues as salinity decreases in the surrounding medium (Mantel and Olson, 1976; Mangum et al., 1978; Saintsing and Towle, 1978; Spencer, 1979; Siebers et al., 1982; Towle, 1984). Towle et al. (1982) found this adaptive response in horseshoe crab coxal gland enzyme but not in preparations of whole gill.

The gill, on the other hand, has long been considered to be the most important osmoregulatory organ in fishes and euryhaline crustaceans (Keys and Willmer, 1932; Croghan, 1958; Philpott and Copeland, 1963; Towle et al., 1976; Pequeux and Gilles, 1978; Girard

and Payan, 1980), and the excretory organ plays no appreciable role. In horseshoe crabs, however, whereas the coxal gland clearly contributes to this regulation (Towle et al., 1982), the contribution of the gill and intestine is believed to be small.

This study examined the ultrastructure of isolated regions of the gill epithelium of the horseshoe crab and tested for the presence of Na^++K^+-ATPase and adaptive activation in response to external salinity changes, the null hypothesis being no response.

MATERIALS AND METHODS

Animals and General Experimental Procedures

In the summers of 1983 and 1984, adult horseshoe crabs (740-1840 gm, 19-22 cm prosomal width) were collected from an oceanside beach in Accomack County on the Eastern Shore of Virginia. Some were held in outdoor running seawater tables (29-32 ppt, ambient temperatures) at the Eastern Shore Laboratory of the Virginia Institute of Marine Science (VIMS) in Wachapreague, Virginia from which they were later collected for experimental uses. In January 1985, adult crabs (850-980 gm, 17-21 cm prosomal width) were obtained from Hampton Roads watermen after being dredged from the Chesapeake Bay. All animals were maintained in aerated 25 gallon Nalgene aquaria equipped with portable filters. Animals held longer than one month were regularly fed clams and squid. Of these, those used in experiments were starved for one week prior to being sacrificed. Salinity of experimental media collected either from Wachapreague (29-32 ppt) or Gloucester Point (16-20 ppt) was adjusted to desired concentrations with tap distilled water and Instant Ocean sea salt and monitored with a YSI model 33 conductivity meter. Water

temperature fluctuated between 16 and 20 C.

Salinity Acclimation

According to Towle et al. (1982) blood osmolality and sodium and chloride ion concentrations reach equilibrium within 24 hours after a salinity decrease in the external medium of not more than 300 mOsm. In the present experiments, individual Limulus destined for low salinity acclimation were allowed at least 36 hours in intermediate salinities. Blood and seawater samples were taken every 12 hours to ensure that steady-state was reached. Blood samples were taken at the base of the telson with a hypodermic syringe. Osmolality was measured with a Wescor vapor pressure osmometer (model 3130A) after 2-minute centrifugation in a Fisher Microfuge. High salinity animals were treated similarly, blood and seawater samples being taken periodically to ensure equilibration before sacrifice.

Electron Microscopy

Lamellae were removed from the gills of horseshoe crabs acclimated to low (15 or 18 ppt, n=2) and high (32 or 35 ppt, n=3) salinities and immediately placed in glutaraldehyde solution (2% in phosphate buffer: 0.1M PO_4, pH 7.2-7.4, with 0.15M sucrose). Upon dissection

into central and peripheral regions, each region was chopped into rectangles approximately 2x4 mm. Eventually, the ventral and dorsal chitinous cell layers composing each lamella were separated resulting in four sections: central dorsal (CD), central ventral (CV), peripheral dorsal (PD), peripheral ventral (PV). (See Figure 1 for an illustration of this dissection.) Sections were fixed for 2 hours in fresh glutaraldehyde solution followed by three 10 minute rinses in phosphate buffer. The tissue was post-fixed for 2 hours in 1% osmium tetraoxide in phosphate buffer, serially dehydrated with acetone and en bloc stained with uranyl acetate for 8 to 24 hours. Tissue was embedded in either SPURRS or EMBED medium. Thin sections were cut on a Sorvall MT 2-B ultramicrotome, stained with lead citrate and examined on a Zeiss EM 9S-2 or EM 109 electron microscope.

Negatives depicting a whole lamella and light microscope views of cross-sections through central and peripheral regions were provided by C.P. Mangum. Tissue had been fixed in Bouin's solution and stained with toluidine blue.

Cuticle Thickness and Blood-Water Diffusion Distance

Measurements of cuticle thickness and blood-water

diffusion distance were made from electron micrographs of cross-sections of lamellae. The largest and smallest distances across the cuticle alone and the cuticle plus epithelium were recorded from each useable micrograph. These estimates should be viewed with caution as they were made from sections of tissue from only one animal with the exception of central ventral measurements which were made from two animals. This was necessary because the other sections were not perpendicular.

Na^++K^+-ATPase activity

Upon acclimation to the desired salinity, all of the gill books were removed from the crab along with a lobe of the coxal gland and in some animals a portion of intestine. The tissue was then placed in ice-cold homogenizing medium containing 0.25 M sucrose, 6 mM disodium ethylenediamine tetraacetic acid, and 20 mM imidazole, pH adjusted to 6.8 with acetic acid (Towle et al., 1982). Initially, the central dark patch was separated from the peripheral region in approximately 20 lamellae from each book and a sample of whole lamellae was taken as well. To test for differences between gill books, after blotting and weighing the tissue was frozen in liquid nitrogen in 5 volumes of fresh homogenizing medium and stored at -70 C until tissue from all five

books could be assayed on the same day. Otherwise, the tissue was homogenized immediately and thoroughly with a hand-held glass homogenizer in 20 volumes of fresh homogenizing medium containing 0.2 volumes of 10% sodium deoxycholate. The homogenate was then filtered through two layers of cheesecloth and assayed immediately. Eventually, as in preparation for electron microscopy (Fig. 1), the central and peripheral regions were separated into their ventral and dorsal sides. Each section was then assayed separately for Na^++K^+-ATPase activity.

Total ATPase activity was measured by an assay system that couples the oxidation of NADH, measured spectrophotometrically at 340 nm, to the breakdown of ATP (Towle et al., 1982). The reaction mixture contained 0.1 mM NADH, 5 mM disodium ATP, 2.5 mM PEP, 20 ul PK/LDH mixture (Sigma 40-7) 20 mM imidazole (pH 7.8), 90 mM NaCl, 10 mM KCl, and 5 mM $MgCl_2$ in a final volume of 2.0 ml. After 5 min incubation at 30 C, the reaction was started with the addition of up to 0.1 ml of filtered homogenate and monitored in a temperature-controlled, recording Bausch and Lomb 700, Beckman DU2, or Beckman DK2 spectrophotometer set at 30 C. Standardization was accomplished with 5-10 ul additions of ADP. Na^++K^+-dependent ATPase activity was

measured by adding the cardiac glycoside ouabain (1 mM final concentration), a specific inhibitor of Na^++K^+-ATPase, to the reaction medium and calculating the difference in rates between inhibited and uninhibited fractions. Enzyme activity is expressed as nanomoles P_i released from ATP per minute per mg protein. Total protein concentration was measured by Bradford's rapid method (Bradford, 1976) using Bio-Rad dye reagent for protein binding and bovine serum albumin as standard.

Total Na^++K^+-ATPase activity per organ was calculated both as specific activity in nanomoles P_i/min-mg tissue (wet weight) multiplied by an estimated average wet weight of tissue in the organ and as specific activity in nanomoles P_i/min-mg protein multiplied by an estimate of total protein per organ. In the coxal gland, total tissue weight was estimated from the average weight of one lobe for the size range of animals used times 8 lobes per animal. Total protein content was estimated from the average protein concentration in 50 mg tissue (wet weight, standard amount used) multiplied by the average weight of the organ. For the gill, total activity based on weight and protein content was determined from activity in the central ventral region alone. Multiplying the average

weight of central ventral tissue per lamella by an approximate number of 1500 lamellae per animal (Mangum, 1982) yields total central tissue weight for the gill. Total protein in the gill was determined as in the coxal gland.

Statistics

To determine the significance of blood osmoregulation, blood osmolalities were compared with seawater osmolalities using a paired t-test (Fig. 5). Differences in cuticle thicknesses and blood-water diffusion distances between regions of the gill lamellae were determined with a one-way analysis of variance (ANOVA) followed by a posteriori testing (LSD). A two-way ANOVA followed by a posteriori testing (LSD) was used to determine differences in enzyme activity between gill books and lamellar regions (Table I) and between salinities and tissue types (Fig. 17). When necessary, enzyme activities were transformed logarithmically to induce equal variation among samples.

RESULTS

Salinity Acclimation

Figure 2 illustrates the relatively weak osmoregulatory ability of Limulus polyphemus (see also Robertson, 1970). Animals were isosmotic to the medium at 30 and 35 ppt and significantly hyperosmotic at 22 and 15 ppt. Among animals subjected to stepwise salinity reductions, most reached hyperosmotic equilbrium within 36 hours although two animals required 48 hours and one animal took 4 days to reach steady state. All five animals acclimated to extra high salinity (38 ppt) were slightly but significantly hypoosmotic.

Morphology

As reported earlier (Mangum, 1982), each lamella has a dark elliptical patch near its center surrounded by a thinner region edged with a thick border of chitin (Fig. 3). Figure 4 amplifies the surface view of cells in the pillar network seen in cross-section in Figure 5. Figures 5 and 6 show the single epithelial layers on each side of the blood space in both regions. The central region sports an epithelium that is thicker and

denser on the ventral side than the dorsal. Pillar cells analagous to those in blue crab gill epithelium (Cioffi, 1984) subtend the blood space throughout the lamellae forming channels for blood flow and girding the chitinous envelopes with structural fibers. The pillar networks in the central region (Fig. 5) appear to have a more well-defined structure than those in the peripheral region (Fig. 6).

Cuticle thickness averages 3.7 um (s.e.=.10, n=18) for the ventral side of the central region, which is significantly larger than 3.4 um (s.e.=.08, n=20) for the ventral side of the peripheral region, both of which are larger than the central dorsal, 3.0 (s.e.=.02, n=8), and the peripheral dorsal, 3.0 (s.e.=.07, n=26) ($P<<.001$). The blood-water diffusion distance averages 4.7 um (s.e.=.31, n=10) in the peripheral ventral region, significantly greater than 3.9 um in both the peripheral and central dorsal regions (s.e.=.10, n=22 and 13, n=8, respectively). The blood-water diffusion distance in the central ventral region averages 9.1 um (s.e.=.24, n=14), significantly greater than all of the others.

The ventral side of the central region is characterized by cells that have mitochondria packed into an extensive network of basal membrane infoldings

and lateral interdigitations (Figs. 7 & 8). These cells are very similar to ion-transporting cells seen in the coxal gland of the horseshoe crab (Towle et al., 1982). Golgi bodies, vesicles and rough endoplasmic reticulum are abundant in the apical cytoplasm (Fig. 9). Mitochondria are present as well but in fewer numbers than in the membrane labyrinth. Nuclei are usually found within the membrane labyrinth. Structures resembling lysosomes occur throughout the cells. An extracellular matrix coats the basal, or blood side of the cell. Hemocyanin molecules give the blood its coarse appearance.

Cells in the pillar network contain structural fibers seen in Figure 10 inserting into the cuticle. As shown here, in the central region pillar cells exhibit the mitochondria-packed membrane labyrinth interwoven with the fibers. These structural organelles appear to be microfilaments like those identified in blue crab branchial epithelia (Cioffi 1984). The lighter, lower portion of the cuticle in Figure 10 is a thicker area that dips down to the pillar network. This structure has so far been seen only on the ventral side, central region. Though not visible here, this reinforcement extends along the ventral side thinning out between pillars as it reaches the peripheral region where it all

but disappears.

In all regions of the lamellae, adjacent cells are commonly attached at their apical borders by septate desmosomes which are sometimes quite extensive. Figure 11 shows one such junction in the peripheral region. Another is visible at much lower magnification in Figure 10. Figure 12 displays the intricate pattern of chitin-plus-protein microfibrils forming a cuticle similar to that in insects (Filshie, 1982). The semi-tangential section exposes an extensive array of pore canals at different levels through the cuticle. At the surface of the cuticle, these structures resemble plant stomata.

The dorsal side of the central region consists of a thin cellular layer lacking ion-transport features (Figs. 13 & 14). Typical cellular organelles such as Golgi bodies, mitochondria, and endoplasmic reticulum can be seen in Figure 13. Nuclei are necessarily elongate (Fig. 14).

A parade of thin cells line the dorsal and ventral sides of the peripheral region (Fig. 15). No ion-transporting cells are seen in this region. When present, nuclei are elongate as in central dorsal cells. In fact, cells in the peripheral region are indistinguishable from those in the central dorsal

region.

No morphological differences were apparent between animals acclimated to low and high salinities in any of the lamellar regions.

Figure 16 shows an arrangement of secretory structures in the peripheral region (similar structures are seen in the central region) that appear to be emptying their contents into a cavity with a common lumen. It is not clear whether these membrane-bound structures are all within a single cell or are each a separate cell. Similar features characterize both mucus-producing goblet cells in the gastrointesticnal tract of vertebrates (Porter & Bonneville, 1968) and secretory cells in the spermatheca of the American cockroach (Smith, 1968).

Na^++K^+-ATPase Activity

Enzyme activity was significantly higher in tissue frozen for up to two weeks than in tissue taken from the same sample and assayed immediately ($P<.001$, paired t-test). In the central region, activity was on the average 2.4 times higher in frozen tissue than in fresh (s.e.=0.68, n=5). In peripheral tissue, activity was 2.2 times higher in frozen than fresh tissue (s.e.=1.09, n=5). Frozen whole lamellae exhibited 5.0

times as much activity as fresh tissue (s.e.=1.92, n=6). This disallowed combining data collected from fresh versus frozen tissue and led to the exclusive use of fresh tissue in subsequent experiments.

At isosmotic salinity (30 ppt), enzyme activity in the central region was similar in each of the five gill books, numbered 1 through 5 anteriorly to posteriorly (Table I). The peripheral region in each of the five gill books likewise contained similar levels of activity (Table I). In whole gill homogenates, book 5 contained significantly lower activity, a trend apparent but not significant in the isolated central region. Variation between books in animals acclimated to anisosmotic salinities was not determined.

In all salinities tested, the central region of the gill lamellae contained higher levels of Na^++K^+-ATPase activity than did the peripheral region with activity in whole undissected lamellae falling proportionately in between (Table I & Figure 17). In the central region as well, separation of the chitinous bilayer into dorsal and ventral halves revealed further physiological differentiation, the ventral side possessing greater activity than dorsal side in all salinities tested (Fig. 17). On the other hand, the low level of activity in the peripheral region of the gill lamellae was not

significantly altered by separation into ventral and dorsal sides. The highest levels of Na^++K^+-ATPase activity were found in the coxal gland and the ventral central patch, the average values of the two not being significantly different.

As evident in Figure 17, no difference in enzyme activity was found between animals with isosmotic blood (30 ppt SW), hyperosmotic blood (15 ppt SW) and hypoosmotic blood (38 ppt SW) in either coxal gland or branchial tissue. The trend of salinity activation is present in the mean values for the central ventral and dorsal regions, but the differences are far from significant due to the very great variability and small sample size. The dorsal side of the central region contained activity levels significantly lower than the ventral side and at high salinity levels not different from peripheral tissue. When the blood was hyperosmotic at 15 ppt salinity, however, activity in central dorsal tissue increased to levels significantly above that in peripheral tissue, the largest change correlated with salinity among tissues tested.

Na^++K^+-ATPase activity in the posterior portion of intestine taken from animals acclimated to very high salinity (38 ppt) was relatively low. (Being located directly below the book gills, the posterior portion of

horseshoe crab intestine is easily accessible once the gills are removed, hence its use.) Unfortunately, no measurement was made of intestine from low salinity animals though data from Towle et al., (1982) indicate a similar level of intestinal enzyme activity (26+1 nanomoles P_i/min-mg protein, n=3) at low salinity (15 ppt).

From an estimated average weight of 150 mg per lobe (8 lobes per animal) and an average specific activity of 5.3 nanomoles P_i/min-mg tissue in animals acclimated to 15 ppt, total Na^++K^+-ATPase activity in the entire coxal gland was estimated to be 6.4 umoles P_i/min. Based on an average protein concentration of 2.4 mg/ml, total Na^++K^+-ATPase activity in the whole coxal gland was estimated to be 5.1 umoles P_i/min. At 38 ppt, average specific activity was 7.9 nanomoles P_i/min-mg tissue and 157.5 nanomoles P_i/min-mg protein. Total coxal gland activity based on tissue weight was 9.5 umoles P_i/min and based on an average protein concentration of 2.6 mg/ml was 9.8 umoles P_i/min. Specific Na^++K^+-ATPase activity in the central ventral region from animals acclimated to 15 ppt averaged 1.8 nanomoles P_i/min-mg tissue. Averaging 3 mg per lamella, the approximately 1500 lamellae comprising the gill contained an estimated total of 8.3 umoles P_i/min, 1.3 times higher than total

activity in the coxal gland based on wet weight. Based on an average protein concentration of 1.6 mg/ml and a specific activity of 85.9 nanomoles P_i/min-mg protein, total Na^++K^+-ATPase activity in the entire gill was estimated at 12.4 umoles P_i/min, 2.4 times higher than that in the coxal gland. At 38 ppt, average specific activity was 1.9 nanomoles P_i/min-mg tissue and 63.9 nanomoles P_i/min-mg protein. Total branchial activity based on tissue weight was 7.4 umoles P_i/min, 1.3 times less than that in the coxal gland. Total branchial activity based on an average protein concentration of 1.7 mg/ml was 9.8 umoles P_i/min at 38 ppt, equal to that in the coxal gland. Total activity in the gill was based on activity in the central ventral region because enzyme activity is apparently masked in homogenates of whole lamellae and intact central region (see Table I and Figure 17).

DISCUSSION

The ability of Limulus polyphemus to regulate its blood osmolytes and do so at least in part by active transport of osmotically important ions has been well established (McManus, 1969, Robertson, 1970, Towle et al., 1982). Whether or not the gill participates in this regulation has until now remained questionable (Towle et.al., 1982; Dunson, 1984). Towle et al., (1982) found that unlike the crustacean antennal gland, the coxal gland in Limulus produces a urine at low salinities (8 ppt) which is hypoosmotic and hypoionic to the blood with respect to Na^+, Cl^-, and Ca^{++}. Excretion of this dilute urine along with hyperosmotic and hyperionic regulation of the blood was accompanied by an increase in activity of the Na^+-transporting enzyme Na^++K^+-ATPase in the coxal gland though not in homogenates of whole gill lamellae. However, much of the activity in whole gill homogenates may have been masked.

Na^++K^+-ATPase activity in whole lamellae both in Towle's study (1982) and this one is much lower than coxal gland activity. However, when lamellae were dissected into their component morphological parts, specific activity in one region (central ventral) was

not different from that in coxal gland. Indeed, the ultrastructure of ion-transporting cells in the two organs are virtually identical (see Fig. 7 and Towle et al., 1982). Based on total Na^++K^+-ATPase activity, the book gills, at least at low salinity (15 ppt), have a slightly greater capacity for osmotic work than the coxal gland. Interestingly, total coxal gland activity in animals acclimated to extra high salinity (38 ppt) is equal to or slightly greater than total branchial activity. Raymond Henry (personal communication) found significant levels of carbonic anhydrase (CA) activity, also thought to play a role in transcellular ion fluxes (for review see Henry, 1984), in both the gill and coxal gland of Limulus. CA activity is much higher in the gill than the coxal gland and dramatically increased with acclimation to either low or very high salinity in both tissues. Exactly paralleling the distribution of Na^++K^+-ATPase activity, CA activity was much higher in the central region than the peripheral region of the gill lamellae and there was no anterior to posterior differentation between books. CA activity and activation with decreasing salinity were almost completely masked in homogenates of whole lamellae, as was probably true for branchial Na^++K^+-ATPase in the study by Towle et al. (1982). Activation of the coxal

gland Na^++K^+-ATPase, which was reported by Towle et al. (1982), was not detected in the present study. One difference in experimental methods was the homogenization technique. Although a glass pestle as used in the present study grinds chitin more thoroughly than a plastic one as used in the earlier study (D.W. Towle, pers. comm.), for homogenizing soft tissue like coxal gland hand-powered homogenization (present study) is probably less effective and certainly less consistent than machine-powered (Towle's study). Another difference was the use of Virginia animals held in a closed system rather than North Carolina animals held in running seawater. While the basis of differences in geographic origin and/or holding conditions is not apparent, it has been noted previously. Virginia animals held in closed aquaria had a much lower tolerance of low salinity and suffered much greater mortality as a result of abrupt transfer to low salinity (Mangum et al., 1976).

In any event, an increase in Na^++K^+-ATPase activity in osmoregulating tissues usually accompanies the onset of anisosmotic regulation, though not always. The blue crab C. sapidus (Towle et al., 1976; Mangum and Towle, 1977; Neufeld et al. 1980), the shore crab Carcinus maenas (Siebers et al., 1982), an estuarine portunid

crab Thalamita crenata (Spencer et al., 1979) and the semiterrestrial fiddler crab Uca pugnax (Holliday, 1985) at least double their branchial Na^++K^+-ATPase activity between 100% and <10% SW. In all of these crabs, Na^++K^+-ATPase activity is higher and undergoes greater activation in posterior gills than in anterior ones.

The possible exceptions to this "activation rule" occur among terrestrial and semiterrestrial species and among fully aquatic species which are relatively weak osmotic regulators. Limulus polyphemus falls in the latter category as a weak hyperosmotic regulator which does not increase branchial Na^++K^+-ATPase activity with decreasing environmental salinity. Metopograpsus thukuhar is a semiterrestrial grapsid which does not increase branchial Na^++K^+-ATPase acitivity when exposed to low salinities even though blood Cl^- concentration is maintained well above that in the external medium (Spencer et al., 1979). The terrestrial coconut crab from Palau, Birgus latro, is able to utilize and withstand exposure to freshwater while maintaining a hyperosmotic blood with a constant level of enzyme activity in its vestigial gill. As in Limulus, however, this level of branchial Na^++K^+-ATPase is relatively high, 85 ± 8 (s.e.) to 107 ± 7 (n=3 or 4). Activity is even higher in the antennal gland and shows adaptive

increase upon exposure to freshwater (Towle, 1981b). Intestinal Na^++K^+-ATPase responds similarly. These two organs possibly assume more important roles in osmoregulation in this lung-breather than they play in more typical crabs.

In addition to physiological mechanisms, terrestrial and semiterrestrial species may regulate hydration and desiccation behaviorally by entering and exiting the medium. The terrestrial anomurans Coenobita and Cardisoma reportedly regulate blood osmolality in this manner (Gross, 1964). Although basically a subtidal species (except during copulation), Limulus resembles the terrestrial coconut crab in that either the level of branchial enzyme activity present in isosmotic salinities is sufficient to produce the slight but significant hyperosmoticity necessary for survival at low salinities or additional mechanisms are employed, such as greater contributions by the excretory organ. Changes in the permeability of cuticle or membrane to water and ions with changing environmental salinity is another possible mechanism (Rudy, 1967; Smith, 1970; Hannan and Evans, 1973; Spaargaren and Mors, 1985) though in adult horseshoe crabs cuticle permeability does not appear to change with acclimation to various environmental salinities (Dunson, 1984).

A morphological as well as physiological spectrum exists among aquatic animals responsive to changes in their environment. In highly efficient osmoregulators like the blue crab, ion-transporting cells in the gill have numerous apical microvilli and extensive basolateral membrane infoldings packed with mitochondria (Copeland, 1964; Towle et al., 1983). Gill epithelial cells in the Chinese crab Eriocheir sinensis are similar to those in the blue crab and, in addition, undergo ultrastructural changes with acclimation to reduced salinity. (Barra & Pequeux, 1984). The apical surface area of the cell decreases due to shortening of apical invaginations. The osmoconforming stone crab, Cancer pagurus does not have any of the specialized salt-transporting features in cells of the gill epithelium which correlates with its inability to control blood ion levels (Pequeux et al., 1984). As a weak hyperosmotic regulator, Limulus is intermediate in transport-related ultrastructure. Ion-transporting cells in the gill of Limulus do not have apical microvilli but do exhibit an extensive labyrinth of presumably Na^++K^+-ATPase-containing basolateral membranes with many closely associated mitochondria.

Unlike brachyuran crustaceans (Neufeld et al., 1980; Siebers et al., 1982; Spencer et al., 1979;

Holliday, 1985; Pequeux and Gilles, 1984), no anterior to posterior functional specialization exists between gill books in Limulus. The Limulus gill does, however, exhibit a division of labor within each lamella similar to that in the blue crab (Neufeld et al., 1980). Not only are the ion-transporting cells and the highest level of enzyme activity found in the central patch, but they are also concentrated on one side of the blood space, the ventral or recovery-stroke side of the lamellae. The specialization of a particular region or of particular gills for osmotic regulation presumably permits the other regions or gills to maintain the thinnest possible epithelium for respiratory gas exchange. This appears to be the case in Limulus. Blood-water diffusion distances in the peripheral region and the dorsal side of the central region are roughly 1/3 to 1/2 the average distance in the central ventral region.

By mounting a lamella in an Ussing chamber with media of various concentrations on either side of the lamella, Dunson (1984) measured translamella ion fluxes in Limulus and found low permeablility to Na^+ and Br^- (0.5-0.6 micromoles/cm^2-hr). Coupling this with the gill's high permeability to water, he concluded that the gill probably was not actively controlling ion movements

across its surface. Having placed the lamellae randomly in the Ussing chamber, however, his results may have been confounded by the morphological and functional differentiation that exists within the gill lamella. Also, because physically separating the double-chitin-layer lamella resulted in very high ion flux rates probably due to structural damage, Dunson necessarily had to base his conclusions on ion movements across the whole double-chitin-layer lamella. Ion fluxes across one layer from SW into blood and vice versa would be a more relevant measure if it could be done without damage to the tissue.

At high salinity the central dorsal region of the gill lamellae is practically devoid of salt-transporting cells and of Na^++K^+-ATPase activity and in fact is quite similar to peripheral tissue. At low salinity, however, enzyme activity in central dorsal tissue becomes significantly greater than that in peripheral tissue. Though not revealed in the present study, this increase in Na^++K^+-ATPase activity may indicate a development of salt-transporting features in cells in that region. In blue crab gill, a significant increase in Na^++K^+-ATPase activity was accompanied by a proliferation of salt-transporting cells from the lateral portion of each lamella to the middle and medial portions with

acclimation to low salinity (Neufeld et al., 1980; Copeland and Fitzjarrell, 1968 as cited in Neufeld et al., 1980).

On the morphological and physiological spectrum, then, the gill of the horseshoe crab falls between that of fully euryhaline osmoregulators and stenohaline osmoconformers, correlating well with the animal's relatively weak ability to regulate blood ions. Although the lack of Na^++K^+-ATPase activation in animals acclimated to hyperosmotic salinity needs further attention, the presence of typical ion-transporting cells and high levels of Na^++K^+-ATPase activity found in certain regions of the lamellae is convincing evidence for a role of the gill in osmotic and ionic regulation in the horseshoe crab.

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Figure 1. Dissection of a gill lamella in preparation for Na^++K^+-ATPase assay and electron microscopy.

a. Ventral view of an adult male horseshoe crab.

b. Removal of gill book #1.

c. Lifting flap to expose lamellae.

d. Removal of individual lamellae.

e. Removal of thick chitinous edge, dissection of central from peripheral regions.

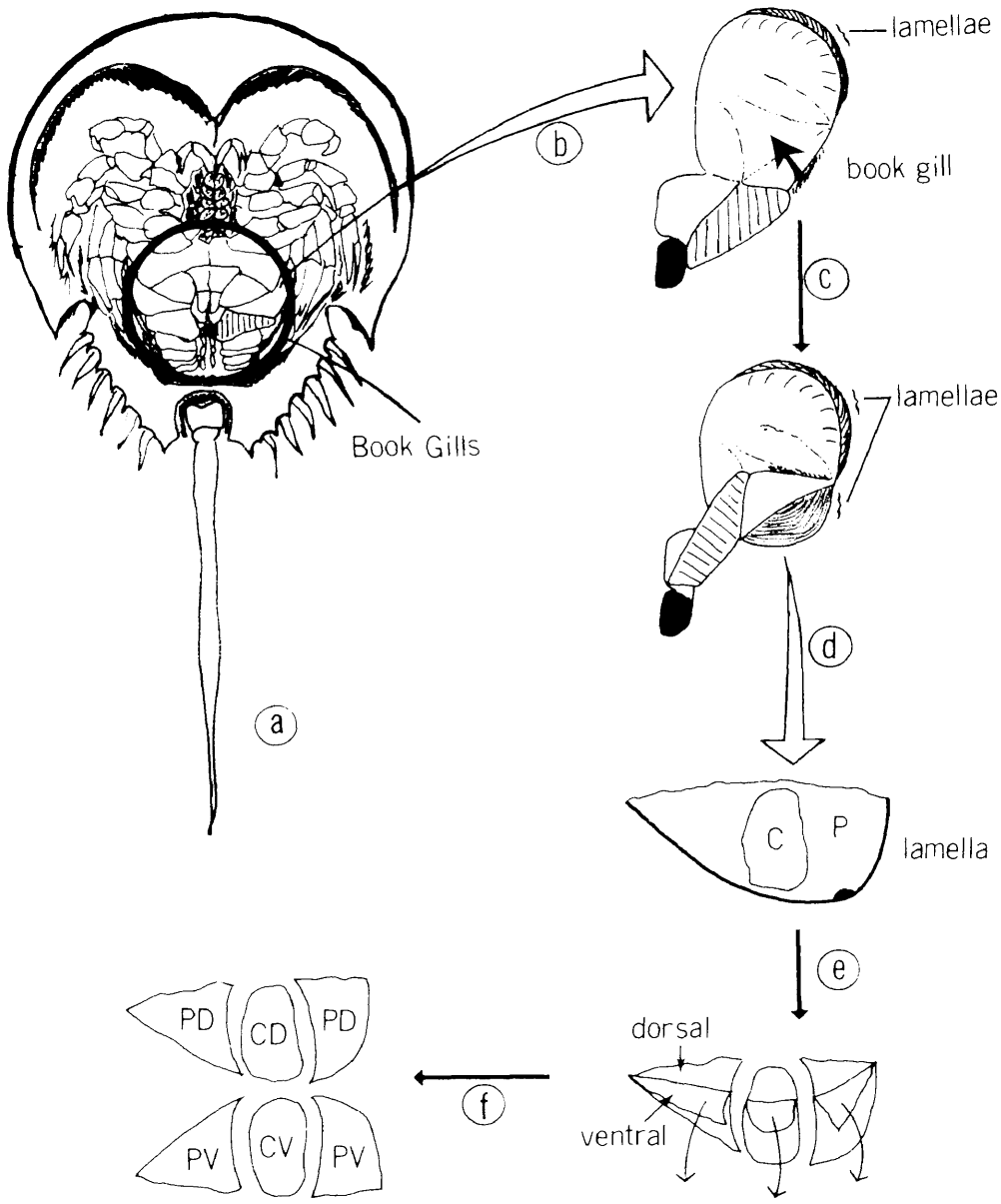
f. Separation of ventral from dorsal sides.

Resulting regions: PD = peripheral dorsal

PV = peripheral ventral

CD = central dorsal

CV = central ventral


lamellae
book gill
b
c
lamellae
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e
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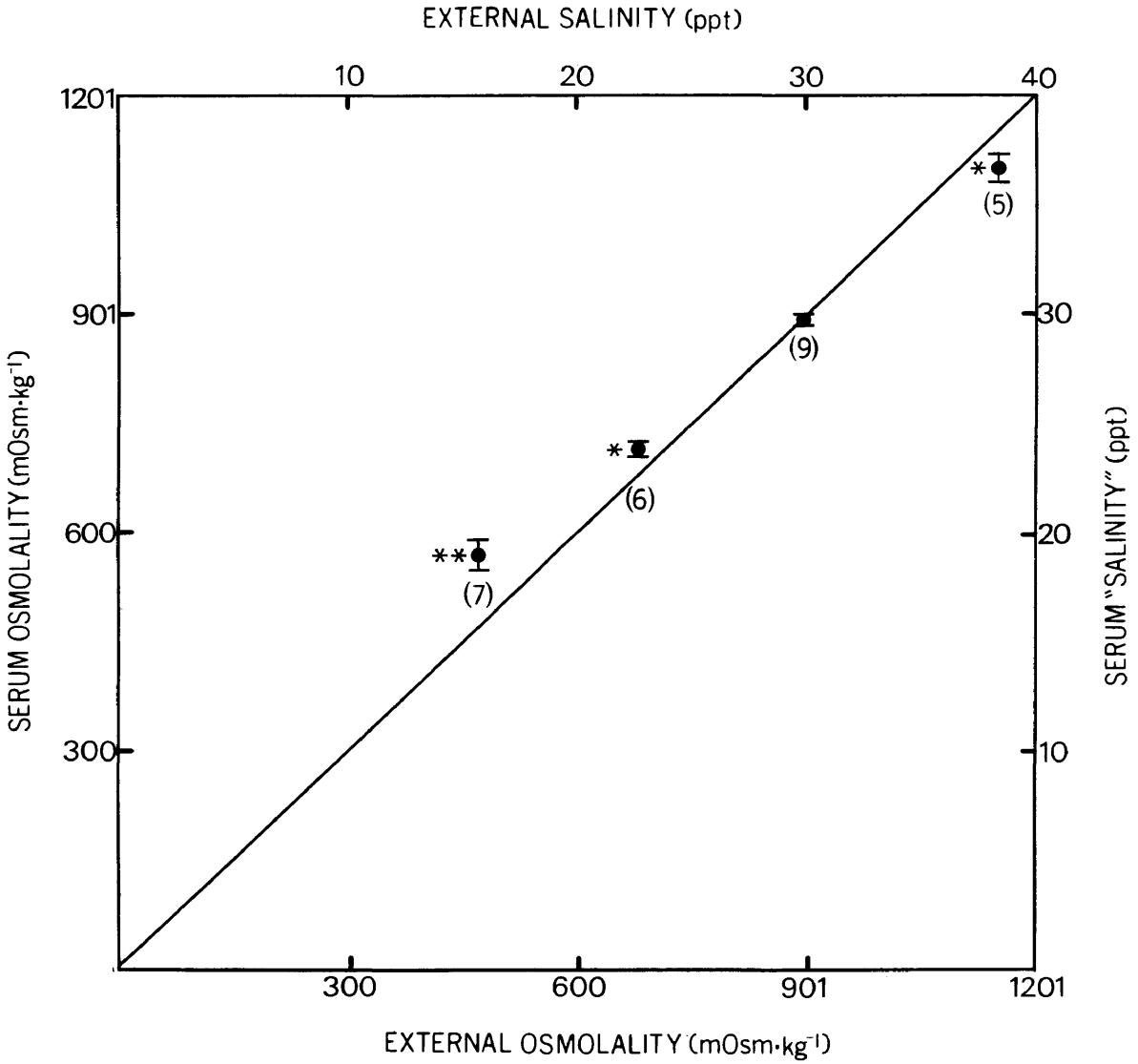


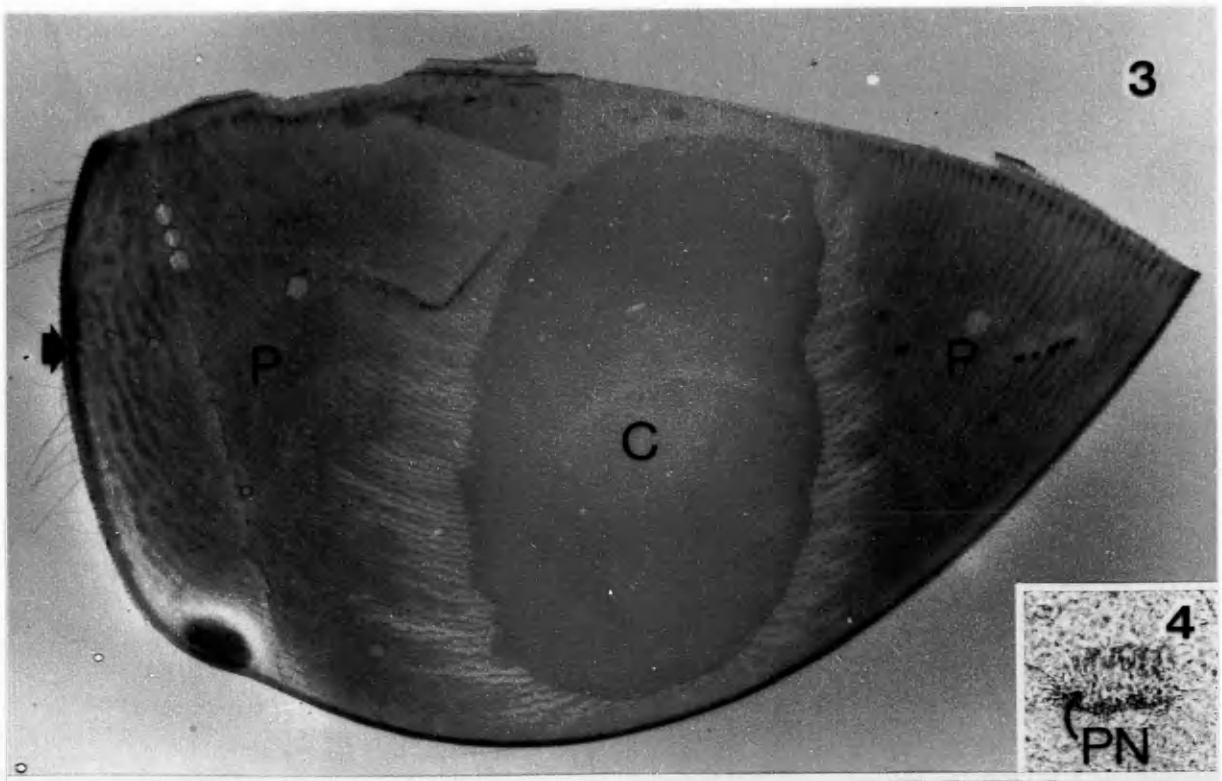
Figure 2. Serum osmolality in relation to external osmolality. Closed circles with bars represent mean ± s.e (n). Asterisks indicate serum-medium differences. *P<.05 **P<.01

Figure 3. Whole mount of a gill lamella (arrow indicates thick chitinous border). C, central region. P, peripheral region. X4.5.

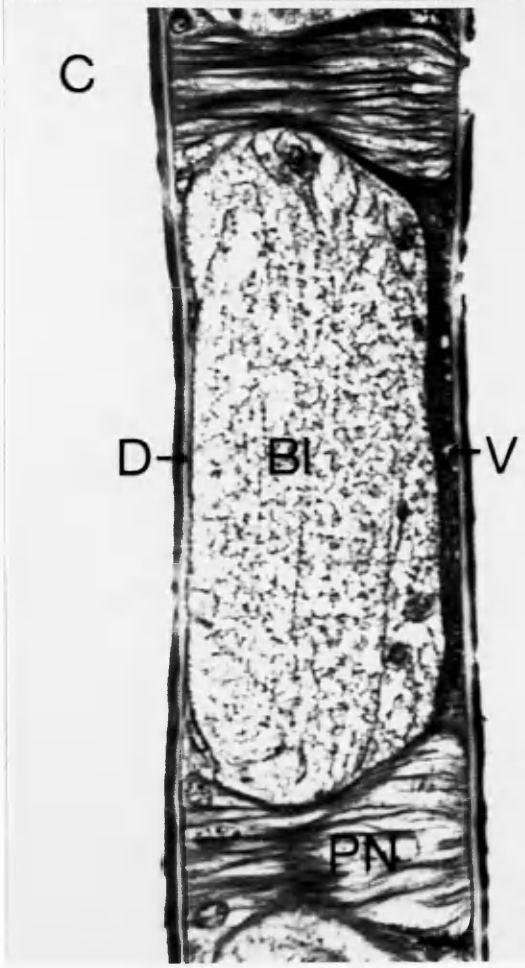
Figure 4. Cuticular surface of the lamella showing a pillar network, PN, visible in longitudinal section in Figures 4 and 5. X236.

Figure 5. Longitudinal section through the central region showing the ventral, V, and dorsal, D, epithelial layers and the fibrous pillar network. Bl, blood. X608.

Figure 6. Longitudinal section through the peripheral region showing the less orderly arrangement of the pillar networks. The ventral and dorsal epithelia are indistinguishable from each other. X383.


3
P
C
P

4
PN


C
D
Bl
V
PN
5

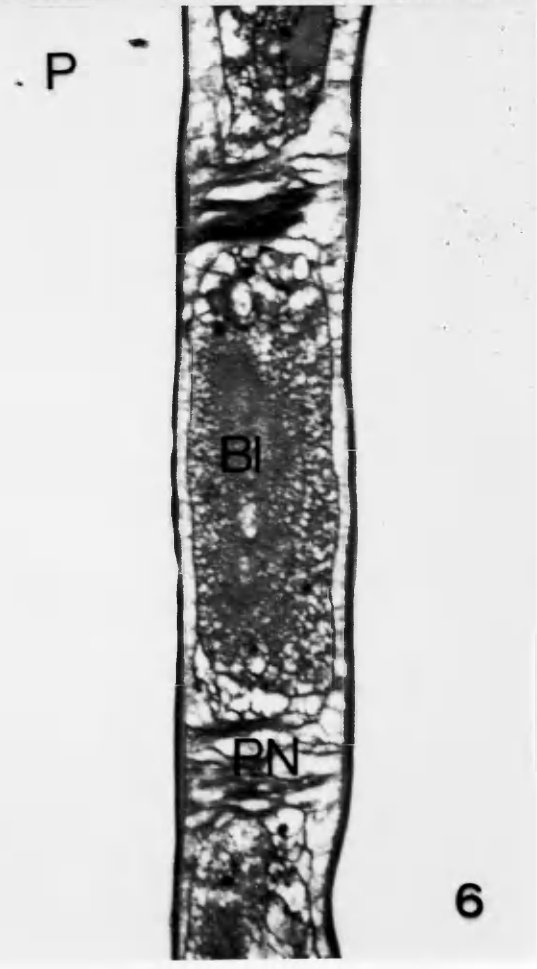

P
Bl
PN
6

Figure 7. Semi-tangential section through an ion-transporting cell on the ventral side of the central region showing the elaborate membrane labyrinth, ML, with densely-packed mitochondria. Nu, nucleus. Cu, cuticle. em, extracellular matrix. X4,515.

Figure 8. Enlarged view of adjacent plasma membranes (arrows) with closely-associated mitochondria (m) of the labyrinth. X103,140.

Figure 9. The apical cytoplasm in an ion-transporting cell. Abundant rough endoplasmic reticulum, er, Golgi bodies, G, and scattered mitochondria are present. X16,243.

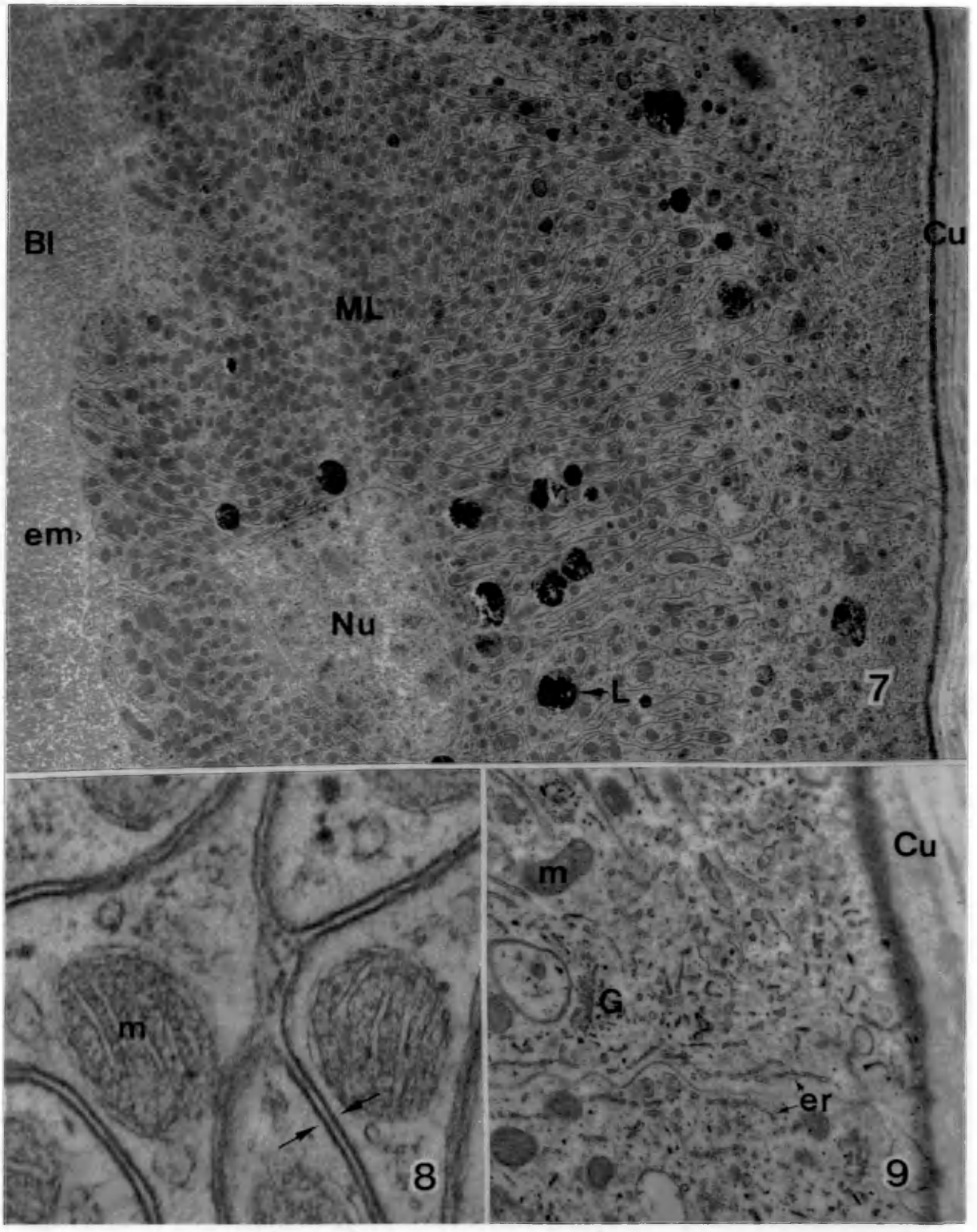

Bl
ML
Cu
em
Nu
L
7
m
8
m
G
Cu
er
9

Figure 10. Cells in a pillar network in the ventral central region. Bundles of microfilaments, Mf, insert into the cuticle (arrows). Septate desmosomes, SD, seen more distinctly in Figure 11, connect adjacent cell membranes. Pore canals, pc, perforate the cuticle. X28,060.

Figure 11. Close-up view of septate desmosomes. Microtubules, mt, are present in the cytoplasm. X185,500.

Figure 12. Semi-tangential section through the cuticle showing pore canals near the surface (*) and further within the cuticle. Chitinous fibrils create the fingerprint mosaic seen here. X34,000

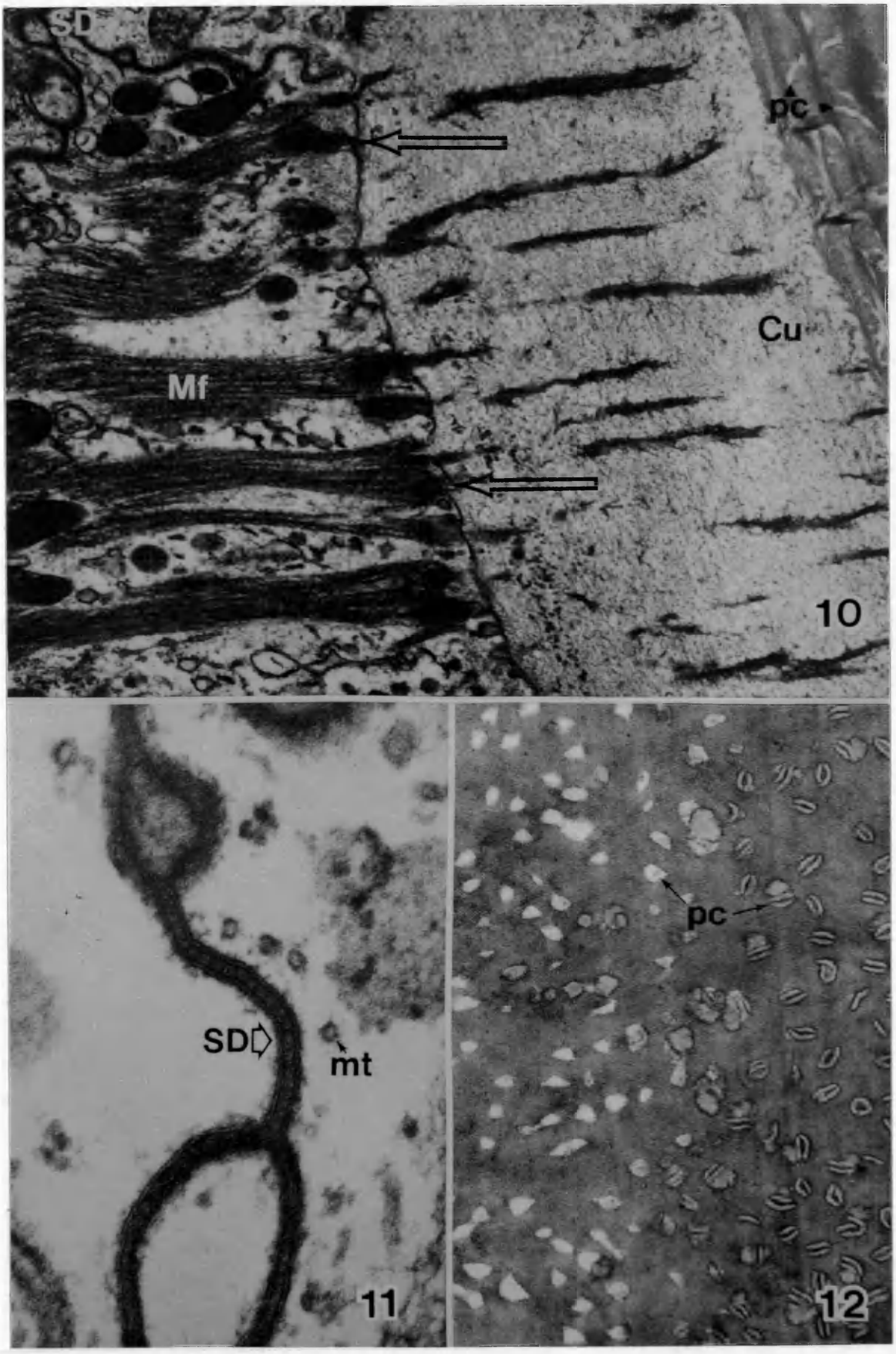

SD
pc
Cu
Mf
10
SD
mt
11
pc
12

Figure 13. Numerous mitochondria and rough endoplasmic reticulum in a central dorsal cell. Mitochondria are usually less concentrated in this region than shown here. X15,840.

Figure 14. Partial view of a flattened nucleus in the central dorsal cell. Secretions appear in the pore canals (arrows). X15,840.

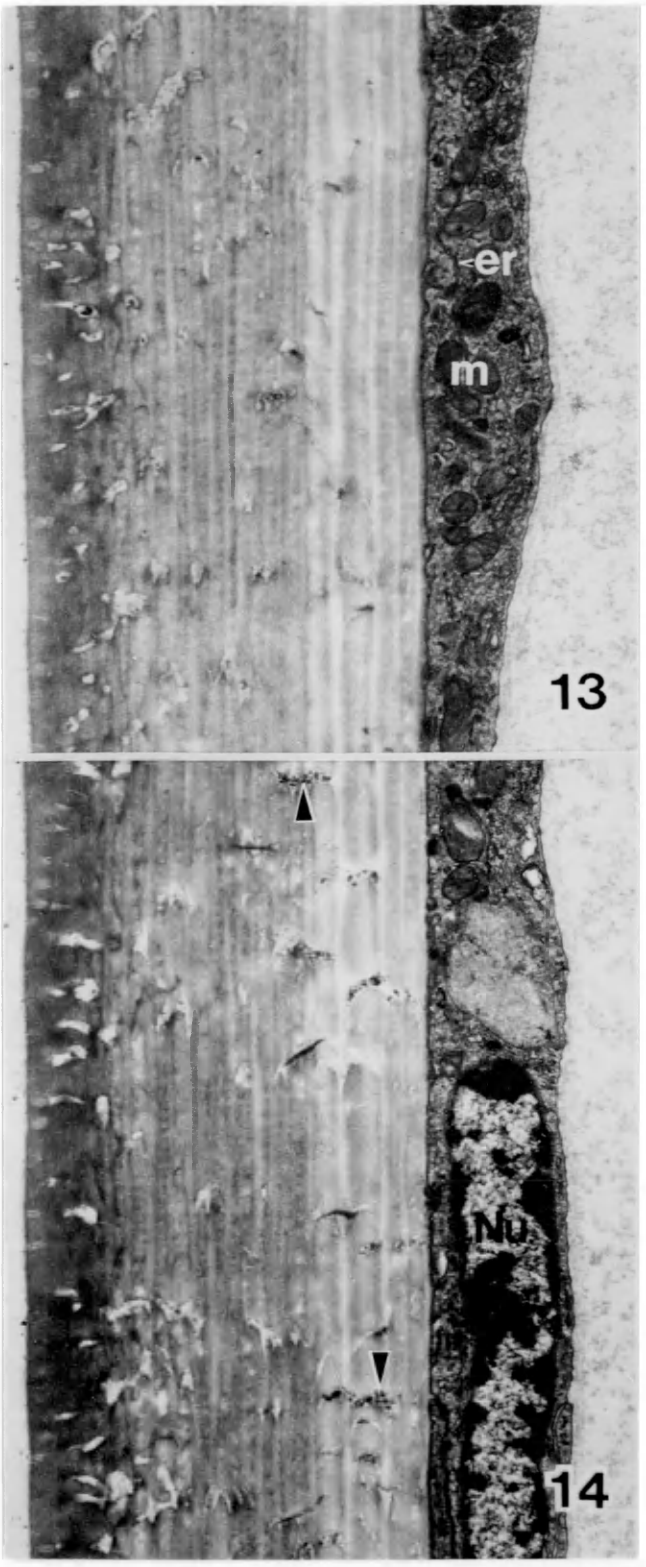
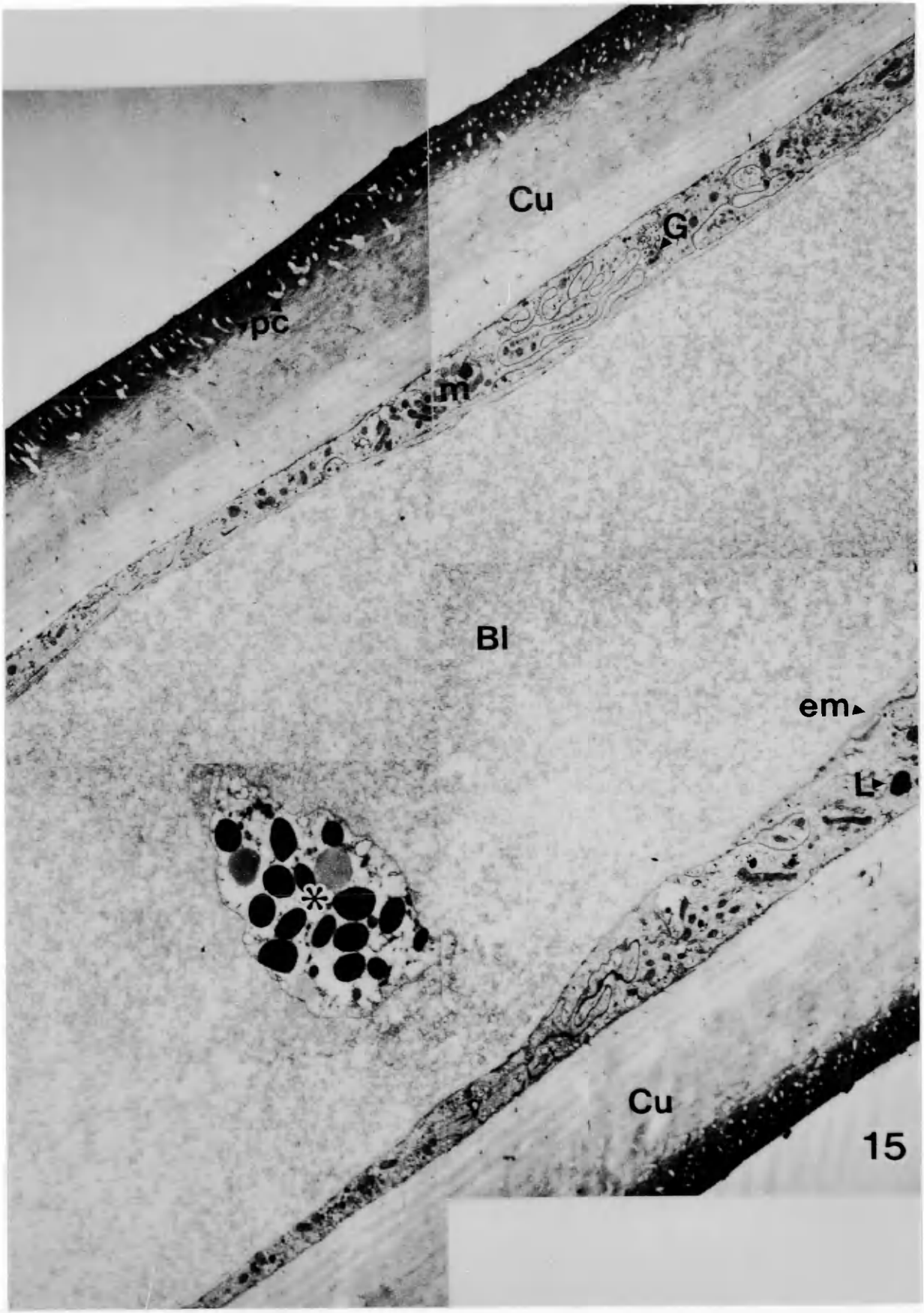

er
m
13
Nu
14

Figure 15. Abbreviated view across the blood space in the peripheral region. Blood-space is reduced ca. 25X relative to the cuticle-cell thickness. Cells on the dorsal and ventral sides are indistinguishable from each other. Typical organelles are present, e.g. mitochondria and Golgi bodies. Pore canals punctuate the exocuticle. An amebocyte (*) in the blood accentuates this composite picture. X5,780.


Cu
G
pc
m
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em
L
Cu
15

Figure 16. An assemblage of suspected secretory cells in the peripheral region. Microvilli, Mv, extend into a cavity (*) at the center of which a common duct-like structure, D, is barely visible. Dilated endoplasmic reticulum (arrow) is interspersed with electron dense bodies (DB) in the cytoplasm. Golgi bodies and numerous vesicles, V, are present as well. X8,840.

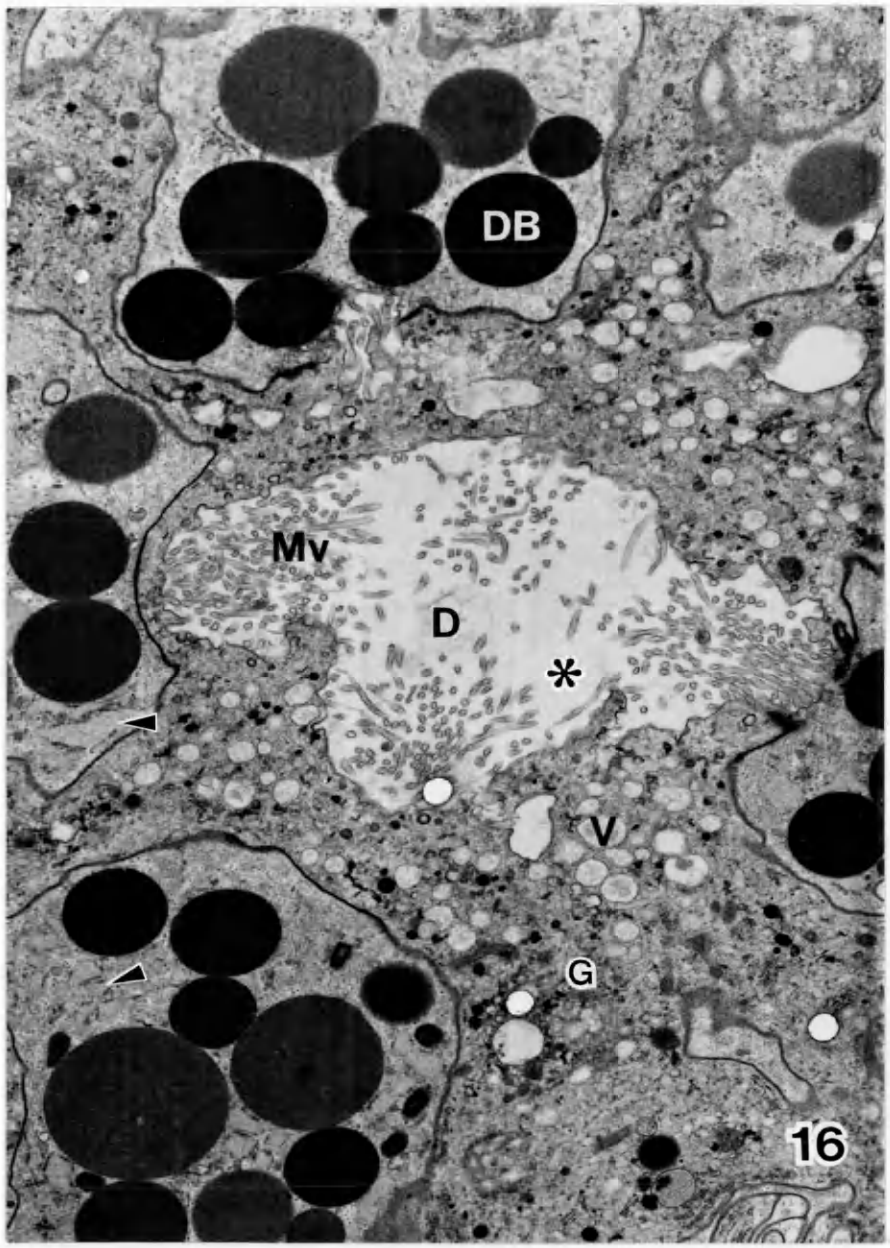
DB
Mv
D
*
V
G
16

Table I

Na++K+-ATPase activity in each of the gill books from *Limulus polypphemus* acclimated to 30 ppt.

Book #	Central	Peripheral	Whole
1	99.8 (20.6, 6)	12.7 (2.9, 6)	41.3 (4.9, 6)
2	96.1 (18.6, 6)	9.2 (4.1, 5)	64.3 (13.0, 6)
3	99.6 (20.1, 6)	10.4 (2.9, 5)	65.2 (10.9, 6)
4	94.3 (18.7, 6)	16.2 (5.2, 6)	50.1 (9.8, 5)
5	65.4 (10.7, 6)	12.2 (1.8, 6)	36.1 (3.7, 6)

Activity is expressed as nanomoles Pi/min-mg protein. Data are presented as mean (s.e., n). Differences between books in the central region and in the peripheral region are not statistically significant. Between regions, activities are different ($P << .001$): Central>Whole>Peripheral

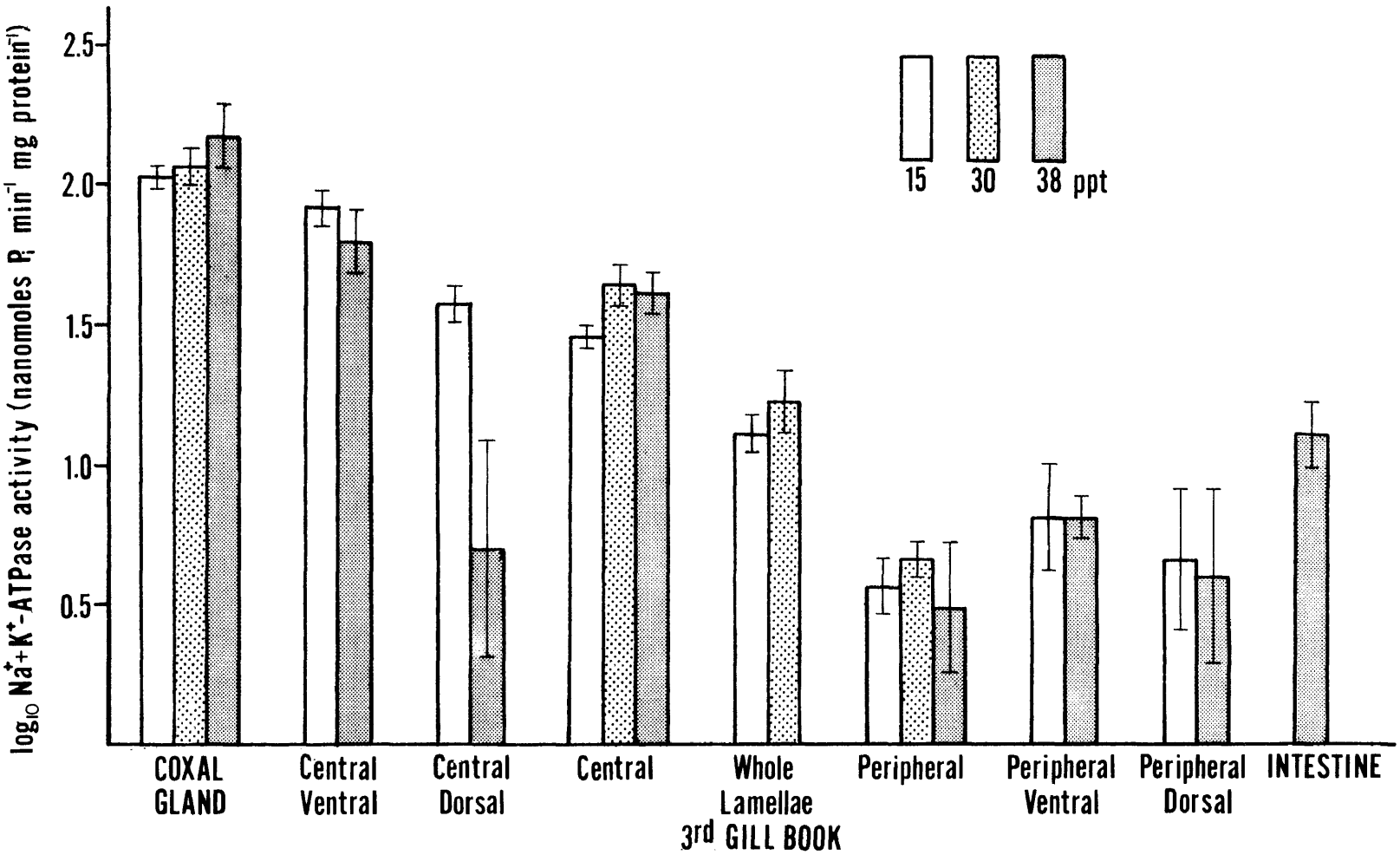

Figure 17. Na^+K^+-ATPase activity in tissues from Limulus polyphemus acclimated to different salinities. Data represent mean ± s.e., $y = \log_{10}(y+1)$.

VITA

Susan Ann Jackson

Born in Sacramento, California, September 4, 1958. Graduated from El Camino High School, Sacramento, California in June, 1976. Received A.A. degree (general education) from American River Community College in Sacramento, California in June, 1978. Attended the University of California, Davis, California from 1978-81, receiving a B.S. in Biological Sciences in June, 1981. Teaching assistant in biology at the College of William and Mary, 1982-84. Candidate for M.A. in Biology at the College of William and Mary, 1982-86. Presently employed with the University of California as a Staff Research Associate at the Bodega Marine Laboratory, Bodega Bay, California.